Published in final edited form as:

Leuk Lymphoma. 2015 May ; 56(5): 1524–1527. doi:10.3109/10428194.2014.963076.

Integrated Analysis of CRLF2 Signaling in Acute Lymphoblastic Leukemia Identifies Polo-Like Kinase 1 as a Potential Therapeutic Target

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Acute lymphoblastic leukemia is the most common cancer in children, and prognosis among adult patients is still poor. Recently, Cytokine Receptor-Like Factor 2 (CRLF2) was identified as a prognostic factor in ~6% of pediatric and adult patients with precursor B cell-acute lymphoblastic leukemia (B-ALL).^{1, 2} In addition, several independent studies also demonstrated that CRLF2 acts as an oncoprotein.^{3–6} The Children's Oncology Group found that high expression of CRLF2 confers an adverse prognosis in high-risk B-ALL patients.⁷ The mechanism of CRLF2-associated leukemogenesis, however, is still unclear. In *CRLF2*-rearranged B-ALL cases, CRLF2 is overexpressed because of genetic rearrangements. Interestingly, about one-half of cases co-harbor an activating mutation in exon 16 of *JAK2*, most commonly involving R683 or I682.^{3, 6} In a murine progenitor B cell line, Ba/F3, it has been shown that the combination of CRLF2 overexpression and *JAK2* R683G confers IL-3-independent growth, implicating their role in triggering leukemic proliferation.^{5, 6} As reflected in phenotypes, the signaling downstream of CRLF2 overexpression and *JAK2* R683G is different from normal CRLF2 signaling.⁸ In this study, we aimed to take

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

advantage of this discrepancy to identify potential therapy targeting this aberrant signaling seen in B-ALL.

To identify kinase inhibitors against leukemic CRLF2 signaling, we subjected Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G to a kinase inhibitor screening system that consisted of 73 kinase inhibitors and other small molecules. An *in vitro* cell proliferation assay was coupled to observe growth inhibition (Figure 1a).⁹ Among all kinase inhibitors, we found Polo-like kinase 1 (PLK1) inhibitor, BI 2536, had the lowest IC₅₀ (11 nM) while the IC₅₀ of p38 mitogen-activated protein kinase inhibitor (VX-745), c-Jun N-terminal kinase inhibitor (JNK II) and Akt kinase inhibitor (GSK-690693) were all 1,000 times higher (Figure 1b). We verified this result by examining if PLK1 is preferentially inhibiting the growth of Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G but not Ba/F3 parental cells nor Ba/F3 cells with BCR/ABL. Another PLK1 inhibitor, volasertib, was used to treat these three cell lines. We found the IC₅₀ of Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G was 9 and 7 times lower than the other two lines, respectively (Figure 1c, 1d and Figure S1). These findings suggest that the growth of Ba/F3 cells conferred by the combination of CRLF2 overexpression and *JAK2* mutant is preferentially inhibited by the PLK1 inhibitor *in vitro*.

To check if Plk1 is downstream of the aberrant signaling by CRLF2 overexpression and *JAK2* mutation, we measured the abundance of Plk1 with immunoblotting. It showed that the expression of Plk1 was higher in Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G than in Ba/F3 parental cells (Figure 1e). Because phospho-Plk1 (Thr210) is the major phosphosite in activated Plk1,¹⁰ we immunoblotted phospho-Plk1 (Thr210) and found it was increased in Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G while Cdk1 was dephosphorylated for cell cycle entry as the downstream effect (Figure 1e). We immunoprecipitated endogenous Plk1 and carried out a non-radioactive kinase assay, which quantified the amount of ATP converted to ADP as result of Plk1 catalytic activity *in vitro*. As shown in Figure 1f, the *in vitro* Plk1 kinase activity from Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G is higher than in Ba/F3 parental cells. Taken together, CRLF2 overexpression and *JAK2* activating mutation can lead to increased expression and activation of PLK1, a finding corroborating the high-throughput kinase inhibition assay as mentioned earlier.

Because PLK1 is a major regulator of centrosomes in mitosis, we chose to study how dysregulation of PLK1 affects cell division. Centrosomes consist of centrioles and pericentriolar material. Within pericentriolar material are PLK1 substrates including γ -tubulin among others.¹¹ At prophase and metaphase, PLK1 can recruit these proteins for centrosomal nucleation of microtubules at their minus ends. To examine PLK1 function for our Ba/F3 system in this regard, we carried out immunofluorescence staining of γ -tubulin, a specific marker for centrosomes. In normal mitosis, centrioles duplicate and summon pericentriolar material to form centrosomes that move toward two poles of the cell. That was our observation in Ba/F3 parental cells (Figure 1g and 1h). Nevertheless, in Ba/F3 cells with CRLF2 overexpression and *JAK2* R683G, we noticed the abnormal separation of centrosomes presented and was surrounded by condensed chromosomes. This would undermine the normal chromosome partition in mitosis. This abnormal appearance of

centrosomes and chromosomes was also phenocopied at the other extreme of the imbalanced PLK1 function where PLK1 was knocked down in SW962 cells.¹² When both PLK1 and its negative regulator, myosin phosphatase-targeting subunit 1 (MYPT1), were knocked down, this mitotic abnormality was rescued. This implicates that either overexpression or knockdown of PLK1 causes its imbalanced function and hence abnormal mitosis. Interestingly, we also found MYPT1 Ser695 was hyperphosphorylated in Ba/F3 cells with CRLF2 overexpression and JAK2 R683G (Figure S2). This would inactivate the function of MYPT1.¹³ Taken together, this aberrant pattern of γ -tubulin in centrosomes could be indicative of PLK1 dysfunction in Ba/F3 cells with CRLF2 overexpression and JAK2 R683G.

Next, we sought to validate the efficacy of PLK1 inhibition *ex vivo*. BI 2536 or vehicle control (DMSO) was used to treat Ba/F3 cells with CRLF2 overexpression and JAK2 R683G, which were then injected into tail veins of sublethally irradiated NOD-scid IL2R γ^{null} (NSG) mice that lacked functional T and B lymphocytes and natural killer cells. Fifteen days after cell injection, necropsy was performed to evaluate cell infiltration in mouse spleens and livers (Figure 2a). While the sizes of mouse spleens and livers from the no injection control were consistent with those of regular NSG mice, all of the mice receiving cell injection had hepatosplenomegaly that could be reduced with the BI 2536 treatment (Figure 2b to 2d). We found Ba/F3 cells with CRLF2 overexpression and JAK2 R683G treated with BI 2536 resulted in significantly smaller spleens (0.5% vs. 1.5% of total body weight, $p < 0.01$) and livers (5.5% vs. 7.6% of total body weight, $p < 0.01$) compared with what untreated cells would do (Figure 2e and 2f). This subsiding of hepatosplenomegaly suggests that the proliferation of Ba/F3 cells with CRLF2 overexpression and JAK2 R683G in mice was thwarted by the PLK1 inhibition, consistent with the result of high-throughput kinase inhibitor assay.

The efficacy of the PLK1 inhibitor in our Ba/F3 cell system led us to examine its effect on human samples. We checked if any different effect of BI 2536 on B-ALL patient samples with CRLF2 overexpression and JAK2 activating mutation compared with wild-type CRLF2 and JAK2. We used two human B-ALL xenografts (CRLF2 overexpression/JAK2 I682F vs. CRLF2 normal expression/JAK2 wild type) that had been passaged in NSG mice to test (Figure 2g). Forty-eight hours prior to culturing the leukemia xenografts, hTERT-transformed human bone marrow stromal cells were seeded as the feeder layer.^{14, 15} Leukemia cells were seeded and treated either with BI 2536 or vehicle control (DMSO). Forty-eight hours later, the leukemia cells were harvested and assessed for viability. In comparison with vehicle control, the viability of CRLF2-rearranged B-ALL cells treated with BI 2536 was lower (48.8% of vehicle) than B-ALL cells without CRLF2 rearrangement or JAK2 mutations (105.3% of vehicle) (Figure 2h). In this particular case of B-ALL, CRLF2 rearrangement was associated with a higher sensitivity to the PLK1 inhibitor.

To summarize, we identified PLK1 as a potential therapeutic target for human CRLF2-rearranged B-ALL with JAK2 activating mutations. From the high-throughput kinase inhibitor screening, *ex vivo* therapy in mouse transplantation experiments and *in vitro* viability assay for human xenografts, inhibition of PLK1 was shown to have a potent activity against Ba/F3 cells with CRLF2 overexpression with JAK2 R683G as well as human

B-ALL cells with CRLF2 overexpression with *JAK2* I682F. In addition, the immunofluorescence imaging implicate that PLK1 is dysregulated downstream of the aberrant CRLF2 signaling. More studies are warranted for understanding the particular mechanism behind these observations, and we suggest future therapy for this subset of B-ALL could be directed against PLK1 and its associated signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by an NIH roadmap grant for Technology Centers of Networks and Pathways (TCNP) (U54RR020839), NCI's Clinical Proteomic Tumor Analysis Consortium initiative (U24CA160036) and a contract (HHSN268201000032C) from the National Heart, Lung and Blood Institute (NHLBI). Thank Mohammad Heydarian for the help on immunofluorescence imaging.

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Figure 1. Inhibition of Polo-like kinase 1 counteracts *in vitro* proliferation conferred by the aberrant CRLF2 signaling in leukemia

- (a) The schematic of the high-throughput kinase inhibition assay.
- (b) PLK1 inhibitor, BI 2536, has the lowest IC_{50} compared to other kinase inhibitors.
- (c) Proliferation inhibition of Ba/F3 parental cells by PLK1 inhibitor, volasertib.
- (d) Proliferation inhibition of Ba/F3 cells with CRLF2 overexpression with *JAK2* R683G by PLK1 inhibitor, volasertib.
- (e) Immunoblotting of Plk1, phospho-Plk1 (Thr210), Cdk1 and phospho-Cdk1 (Tyr15) in Ba/F3 parental cells and Ba/F3 cells with CRLF2 overexpression with *JAK2* R683G.

(f) *In vitro* kinase assay showing that the catalytic activity of immunoprecipitated endogenous Plk1 was higher in Ba/F3 cells with CRLF2 overexpression with *JAK2* R683G than in Ba/F3 parental cells.

(g–h) Immunofluorescence staining of γ -tubulin shows that (g) a normal centrosome before duplication and relocation to two poles of the cell; (h) in Ba/F3 cells with CRLF2 overexpression with *JAK2* R683G, failed partition of centrosomes were surrounded by condensed chromosomes.

Figure 2. Inhibition of Polo-like kinase 1 counteracts the growth mediated by aberrant CRLF2 signaling *ex vivo* and the proliferation of CRLF2-rearranged precursor B acute lymphoblastic leukemia *in vitro*

(a) The strategy of *ex vivo* PLK1 inhibitor therapy involving NOD-scid IL2R γ^{null} (NSG) mice.

(b–d) Comparison of spleen and liver weights between different treatments for Ba/F3 cells with CRLF2 overexpression with JAK2 R683G.

(e) The sizes of spleen and liver in regular NSG mice.

- (c) The sizes of spleen and liver in NSG mice receiving injection of Ba/F3 cells with *CRLF2* overexpression with *JAK2* R683G without BI-2536 pretreatment.
- (d) The shrinkage of the spleen and liver in NSG mice receiving injection of Ba/F3 cells with *CRLF2* overexpression with *JAK2* R683G after BI-2536 pretreatment.
- (e–f) Comparison of spleen and liver weights normalized to body weights from NSG mice with different treatments.
- (g) The schematic of *in vitro* viability assay for human xenografts.
- (h) Human B-ALL cells with *CRLF2* overexpression with *JAK2* I682F tended to be more sensitive to BI 2536 compared to those with wild-type *CRLF2* and *JAK2*.